In leukemia, knock-down of the death inducer-obliterator gene would inhibit the proliferation of endothelial cells by inhibiting the expression of *CDK6* and *CCND1*

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Abstract

Background: Endothelial cells (ECs) are a critical component of the hematopoietic niche, and
the cross-talk between ECs and leukemia was reported recently. This study aimed to determine
the genes involved in the proliferation inhibition of endothelial cells in leukemia.

Methods: Human umbilical vein endothelial cells (HUVEC) were cultured alone or cocultured
with K562 cell lines. The GeneChip assays were performed to identify the differentially
expressed genes. The Celigo, MTT assay and flow cytometric analysis were used to determine
the effect of RNAi DIDO on cell growth and apoptosis. The differently expressed genes were
verified by qRT-PCR (quantitative real-time PCR) and western-blot.

Results: In K562-HUVEC co-cultured cell lines, 323 down-regulated probes were identified and
the extracellular signal-regulated kinase 5 (ERK5) signaling pathway was significantly inhibited.
Among the down-regulated genes, the *death inducer-obliterator gene* (*DIDO*) is a part of the
centrosome protein and may be involved in cell mitosis. As shown in the public data, leukemia
patients with lower expression of *DIDO* showed a better overall survival (OS). The HUVEC
cells were infected with *shDIDO* lentivirus, reduced expression, inhibited proliferation, and
increased apoptosis was observed in *shDIDO* cells. In addition, the expression of *Cyclin-
Dependent Kinase 6* (*CDK6*) and *Cyclin D1* (*CCND1*) genes was inhibited in *shDIDO* cells. At
last, the public ChIP-seq data were used to analyze the regulators that bind with *DIDO*, the
H3K4me3 and PolII (RNA polymerase II) signals were found near the Exon1 and exon2 sites of
DIDO.

Conclusion: The knock-down of *DIDO* will inhibit the proliferation of endothelial cells in the
leukemia environment. The expression of *DIDO* may be regulated by H3K4me3 and the
inhibition of *DIDO* may lead to the down-regulation of *CDK6* and *CCND1*. However, how
DIDO interacts with *CDK6* and *CCND1* needs further study.

Introduction

The increased number of circulating endothelial cells (ECs) in the peripheral blood was detected
in multiple myeloma [1], myelodysplasia [2], and acute myeloid leukemia patients [3]. Besides, the
higher level of circulating ECs and endothelial precursor cells (EPCs) was associated with more
aggressive disease and shorter survival [4] in chronic lymphocytic leukemia.

The cross-talk between leukemia cells and endothelial cells was reported recently. The
endothelial cells (ECs) provide a fertile niche that will promote the proliferation of primitive and
aggressive leukemia cells [5]. Besides, ECs could elaborate on angiocrine factors, which will take
part in the reconstitution of normal and malignant stem/progenitor cells [6]. ECs provide critical
support for the survival and progression of leukemia stem cells (LSCs) [7], which would promote
regeneration of leukemia [8, 9]. On the other hand, the leukemic blasts could secrete numerous

74 cytokines, which will augment the proliferation of microvascular endothelial cells in primary
75 acute myelocytic leukemia (AML) cells [10]. Leukemia may induce the activation of resting ECs
76 and these activated ECs would protect the leukemia cells from chemotherapy injury [11]. Due to
77 the protective effect of ECs in leukemia microenvironment [12], vascular targeted drugs may be a
78 new strategy for AML treatment decisions.
79
80 Leukemia-derived ECs may originate from bone marrow-derived hemangioma blast progenitor
81 cells [13], as the BCR-ABL fusion transcript in ECs was found derived from bone marrow
82 progenitor cells [5]. Besides, the AML cells could integrate into vasculature and fuse with ECs in
83 *vivo*, and the AML cells could differentiate into endothelial-like cells *in vitro* [14].
84 A better understanding of the interaction between ECs and leukemia may inspire the design of
85 innovative therapies for leukemia. Niche target treatment may help restore damaged vascular
86 microenvironment, increase chemotherapy delivery and increase treatment responses. To
87 determine the targets which will inhibit the ECs may be a new direction. In this study, we tried to
88 investigate the genes involved in the interaction between the ECs and leukemia cells by
89 GeneChip. The proliferation of HUVEC cells was inhibited when co-cultured with the K562
90 cells. A *death inducer obliterator gene* (*DIDO*) showed lower transcript abundance in HUVEC-
91 K562 co-cultured cells. Although we have not found a report about the mechanism of *DIDO* in
92 leukemia, there were reports showing that *DIDO* was involved in the development of solid
93 tumors, such as bladder cancer [15], RCC [16], and Melanoma [17]. In this study, we investigated the
94 role of *DIDO* in endothelial cells in the leukemia environment.
95

96 Materials & Methods

97 **Cell lines and cell culture**
98 Human umbilical vein endothelial cells (HUVEC) and Human myeloid leukemia cell line (K562)
99 were purchased from the American Type Culture Collection (Rockville, MD). The cell lines
100 were maintained using RPMI (Roswell Park Memorial Institute) 1640 medium (Gibco Co, USA)
101 supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2. The
102 HUVEC and K562 cell lines were mixed and co-cultured for 4 days. CCK-8 Cell Counting Kit-8
103 (CCK-8) was used to determine the cell viability by colorimetric assays at 450nm [18].
104 For RNA extraction and analysis of cell proliferation and apoptosis, the suspension of K562 cells
105 was removed. Then the HUVEC cells in the two groups (HUVEC vs. HUVEC-K562) were
106 washed by PBS buffer and collected for further experiments. The HUVEC cells were digested
107 with pancreatin before RNA extraction.
108

109 **Plasmid constructs and transfection**
110 For gene knockdown, the GV115 vector was used in this study, which used the green fluorescent
111 protein as a reporter gene, and the multiple cloning sites were driven by a human U6 promoter.
112 The *DIDO* was targeted by the shRNA sequences of 5′- GGATGAGACTCATTCAGAA- 3′. The
113 sequence was cloned into the multiple cloning sites by restriction enzyme of AgeI and EcoRI.

114   Plasmid transfection was performed as a former study [19]. The cell lines were seeded into 96-
115   well plates. After transfection for 2~3 d, the GFP was observed under a fluorescence microscope.
116   The cells were used for further studies when the cell density in the wells reached 70-90%.
117   **Celigo and MTT assays**
118   Cells were inoculated into the 96-well plates (2000 cells / well) and three repeats were taken.
119   The cell numbers were measured by Celigo Imaging Cytometer [19] and the numbers were
120   recorded for 5 days. The cell numbers were normalized to the cell numbers on the first day after
121   seeding.
122   MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay [20] was performed
123   to analyze the proliferation of cells. 1 mg / mL MTT was added to each well and incubated at
124   37°C for 4 hours. Then the culture medium was removed and the DMSO (150 μl) was added into
125   each well, and then the plate was shaken for 3 minutes. The Tecan Infinite M2009PR plate
126   reader was used to measure the absorbance at 490 nm / 570 nm.
127   **Cell apoptosis analysis**
128   The cell apoptosis was measured following the manufacturer's instructions of Annexin-FITC
129   Apoptosis Detection Kit (BD Biosciences, Franklin Lake, NJ, U.S.A.). Cells were cultured in a
130   96-well plate for 3-5 days in the 37 °C incubator, and then the cells were harvested and washed
131   in PBS. Cells were added to 0.5 ml binding buffer and Annexin V-FITC, then the cells were
132   stained in the dark for 15 min at room temperature. Cells stained by Annexin V-FITC were
133   considered apoptotic cells [21] which were measured by a BD Accuri ™ C6 flow cytometer (BD
134   Biosciences).
135   To analysis the cell apoptosis, Caspase 3/7 enzyme activity was measured by Caspase-Glo® 3/7
136   Assay (Promega, G8091). Caspase-Glo 3/7 reagent was added to the sample with a volume ratio
137   of 1:1, and the cells were incubated for another 1 h at 37 °C. The Tecan Infinite M2009PR plate
138   reader was used to detect the luminescence in each well at 490 nm/520 nm [22].
139   **Angiogenesis analysis**
140   The serum-free supernatants of tumor cells from different experimental groups were collected
141   and suspended the HUVEC cells to 2×10^4 cells / 100uL. After being cultivated at 37°C for 4-6 h,
142   the angiogenesis assay was performed by the Celigo instrument.
143   **Microarray processing and data analysis**
144   The samples were hybridized with the GeneChip microarrays (901838, Affymetrix) to determine
145   gene expression abundance according to the manufacturer's instructions. The expression profile
146   was preprocessed by the Limma package in Bioconductor.
147   A robust multiarray averaging algorithm was used to perform background correction, quantile
148   normalization, and probe summarization on the microarray data to obtain a gene expression
149   matrix. The cut-off for the background correction was 20%, and the coefficient of variation was
150   25%. The Benjamini-Hochberg method was used to correct the significant difference level
151   (FDR). The screening criteria for significantly different genes were: |Fold Change| > 1.5 and
152   FDR < 0.05 [23]. The biological pathways analysis of genes was performed by Ingenuity Pathway
153   Analysis (IPA).

RNA extraction and qRT-PCR analysis

According to the manufacturer's protocol, the Trizol reagent (Invitrogen) was used to extract total RNA from frozen cells. For cDNA synthesis, 1 μg of total RNA was used to synthase the cDNA by the Go Script reverse transcription system (Promega, Maddison, MA, U.S.A.). The genes were detected by the SYBR Master Mixture (DRR041B, Takara) using the LightCycler480 Real-Time PCR system (Roche). For qRT-PCR, the GAPDH gene was used as endogenous control. The primers sequences and the length of the amplifications were shown in Table S1. The $2^{-\Delta\Delta Ct}$ method was used to calculate the fold change for gene expression relative to the control.

Protein extraction and Western-blot analysis

Total protein was isolated from cells using protein cell lysis buffer and extracted by centrifugation at 13000 rpm for 20 min at 4°C. The equal amount of whole cell lysate was separated by SDS-PAGE gel electrophoresis. After the proteins were transferred to the PVDF membranes (Bio-Rad, CA, U.S.A.), the membranes were blocked by 5% skimmed milk and immunoblotted with the primary antibodies at 4°C. Then the membranes were blotted with the secondary antibodies at room temperature for 1 h. The following primary antibodies were used: anti-DIDO (1:1000, HPA049904, Sigma), anti-CCND1 (1:500, Cat2978, CST), anti-CDK6 (1:500, Cat3136, CST), anti-GAPDH (1:2000, Sc-32233, Santa Curz). The secondary antibodies were anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology). The Dyne ECL STAR Western Blot Detection kit (Dyne Bio, Seoul, Korea) and a chemiluminescent image system (Fusion Solo system, Villber Lourmat) were used to analyze the protein abundance.

Statistical analysis

The data were shown as the mean ± S.D. from 3 independent replicates. The student's t-test was performed to analyze the quantitative data. $P < 0.05$ was considered statistically significant.

Results

GeneChip microarrays analysis of HUVEC and K562-HUVEC co-cultured cell lines

In this study, the human umbilical vein endothelial cells (HUVEC) were used as endothelial cells models *in vitro*. When the HUVEC cells were co-cultured with the K562 leukemia cell lines for 4 days, the proliferation was inhibited significantly (Figure S1). Then we analyzed the gene expression changes in HUVEC cells when co-cultured with the K562 leukemia cell lines by GeneChip, to investigate the genes which will inhibit the endothelial cells' proliferation in leukemia progression.

Compared with HUVEC lines, 398 probes up-regulated expression and 323 probes down-regulated expression in K562 co-cultured HUVEC lines (Figure S2A). Ingenuity Pathway Analysis (IPA) found that the extracellular signal-regulated kinase 5 (ERK5) signaling was significantly inhibited (Z-score = -2.111) (Figure S2B). On the other hand, the DEGs were mainly enriched in microtubule dynamics (Z-score = 2.783), migration of brain cancer cell lines

194 (Z-score = 2.549), liver tumor (Z-score = -2.782) and cell death of mononuclear leukocytes (Z-
195 score = -2.561) (Figure S2C).
196
197 **Construction of RNAi cell lines and cell proliferation analysis**
198 We selected the first 30 down-regulated expression genes (log2 (change fold) > 1, P < 0.05) for
199 further analysis (Table S2). RNAi lentiviral vectors for these 30 genes were constructed and
200 transfected into HUVEC cells. 22 transgenic cell lines were successfully obtained, including the
201 negative control (NC) and positive control (PC). The cell count results showed that the
202 proliferation of cells was normal in the NC group, and which was significantly inhibited in the
203 PC group. The proliferation folds on the fifth day were 12.09 and 2.12 times higher than those on
204 the first day in the NC and PC groups, respectively. The fold change (FC) of cell count ([FC in
205 NC group on the 5th day compared to which on the 1st day] / [FC in experiment group on the 5th
206 day compared to which on the 1st day]) was used to evaluate the influence of gene RNAi in cell
207 proliferation. The proliferation of *shDIDO*, *shZC3H18*, and *shSMURF2* cell lines was
208 significantly inhibited, and the change fold was 3.37, 2.54, 2.07, respectively (Figure S3).
209
210 **Patients with lower transcript abundance of DIDO showed a better overall survival**
211
212 To determine the effect of the *DIDO*, *ZC3H18*, and *SMURF2* in Leukemia patients, we analyzed
213 the survival based on their expression status from the public data (http://gepia2.cancer-pku.cn/).
214 As shown in Figure 1A, the acute myeloid leukemia (AML) patients with the lower *DIDO*
215 expression level, showed a better overall survival (HR=1.9; P=0.025). However, the different
216 expressions of *ZC3H18*, and *SMURF2* did not affect the overall survival in AML patients
217 (Figure 1B and C).
218
219 **The proliferation of *shDIDO* cell line is inhibited and the apoptosis is increased**
220
221 To further investigate the function of the *DIDO* (Death inducer obliterator) gene in endothelial
222 cells, we analyzed the proliferation and apoptosis of *shDIDO* cells. Firstly, the expression of
223 *DIDO* in *shDIDO* cells was analyzed. qRT-PCR found that the expression level of the *DIDO*
224 gene at the mRNA level was suppressed in *shDIDO* cell lines (P < 0.05), and the reduction
225 efficiency reached 95.1% (Figure 2A). Western-blot detection found that the expression of DIDO
226 protein in the *shDIDO* cells decreased by four times, compared with the *shCtrl* group (Figure
227 2B).
228 The proliferation rate of shDIDO cell line was analyzed by Celigo (Figure 2C and D) and MTT
229 (Figure 2E), and the proliferation rate of the *shDIDO* cells was significantly decreased. This may
230 indicate that the *DIDO* gene is significantly related to the proliferation ability of HUVEC cells.
231 The number of cells in the apoptotic state was detected by Annexin V-APC single staining
232 method, and it was found that apoptosis cells in *shDIDO* group increased significantly than the
233 HUVEC cells (P < 0.05) after 5 days (Figure 2G). Additionally, by detecting the activity of

234 caspase, it was found that the activity of caspase3/7 in the *shDIDO* group was significantly
235 increased. These results indicate that the *DIDO* gene was significantly related to the apoptosis of
236 HUVEC cells (Figure 2H).
237 Due to the importance of angiogenesis in tumor progression, we analyzed the effect of *DIDO*
238 gene depletion on angiogenesis. The ability of *shDIDO* cells to form lumens was analyzed to
239 investigate the metastasis ability of tumors. It was found that the area of angiogenesis-related
240 blood vessels in the *shDIDO* group were 20% less than that in the shCtrl group ($P < 0.05$)
241 (Figure 2F), which indicates that the *DIDO* gene may not associated with HUVEC cells
242 angiogenesis.
243
244 **GeneChip analysis of shDIDO and shCtrl cell lines**
245 To investigate the biological pathways *DIDO* involved, the GeneChip expression profiles of
246 *shCtrl* and *shDIDO* cell lines were analyzed. It was found that 521 genes in the *shDIDO* cells
247 were up-regulated and 1006 genes were down-regulated (Fold Change > 1.5 and FDR < 0.05),
248 compared with *shCtrl* cells (Figure 3A, Table S3). IPA analysis found that the ERK/MAPK
249 signaling was significantly inhibited (Z-score = -2.041) (Figure 3B). Additionally, the functions
250 including morbidity or mortality (Z-score = 6.734) and organismal death (Z-score = 6.709), were
251 significantly activated. The functions including cell viability (Z-score = -5.369), cell survival (Z-
252 score = -5.349) were significantly suppressed (Figure 3C and Table S4).
253 The genes down-regulated in *shDIDO* cells, and which are involved in tumorigenesis and
254 development, were selected for further analysis (Table S5). Among them, 30 probes were
255 identified by qRT-PCR as their expression pattern were similar to the GeneChip (Table S6).
256 DIDO is a part of the centrosome protein and plays an important role in spindle assembly, so we
257 infer that the *DIDO* gene may correlate with the cell cycle. We further analyzed the cell cycle
258 regulation genes of *Cyclin Dependent Kinase 6* (*CDK6*) and *Cyclin D1* (*CCND1*). The
259 transcription of *CCND1* and *CDK6* in the *shDIDO* cells were 0.364 and 0.404 times of the *shCtrl*
260 group, respectively. Meanwhile, the western-blot analysis found that the protein expression
261 levels of CCND1 and CDK6 in *shDIDO* cells were reduced by 81.3% and 58.1%, respectively.
262 However, the DIDO maybe not interact with CDK6 or CCND1 directly, as shown in Figure 3D.
263 We searched the protein interaction database and did not find the direct interaction of DIDO with
264 *CDK6* or *CCND1* either. The genes that may directly interact with *DIDO* were *SRSF1*, *SRPK2*,
265 *EED*, and *WWP2* in the interaction network of *DIDO* analyzed by IPA.
266
267

Discussion

269 Endothelial cells provide a fertile niche that allows for the propagation of primitive and
270 aggressive leukemic clones. This study aimed to identify the genes involved in the interaction
271 between endothelial cells and leukemia.
272 Firstly, the GeneChip assay showed that the expression of 323 probes was down-regulated in
273 K562-HUVEC co-cultured cells (Figure S3A), and the ERK5 signaling was significantly

274   inhibited. It has been reported that the ERK5 pathway mediates cell survival, apoptosis, and
275   proliferation signaling in embryonic stem cells [24]. We infer that the decreased ERK5 signaling
276   may correlate with the proliferation inhibition of HUVEC cells. There is increasing evidence to
277   indicate that the ERK5 signaling takes part in the development and progression of several types
278   of cancers, including breast cancer, myeloma, lymphoma, leukemia [25]. In addition, some studies
279   suggested that the ERK5 could represent a promising target for therapeutic intervention in
280   leukemia [26].
281   To investigate the key genes involved in the inhibition of HUVEC cells when co-cultured with
282   K562, RNAi cell lines of the top 30 down-regulated expression genes were constructed and we
283   analyzed the proliferation of them. The proliferation of *shDIDO*, *shZC3H18*, and *shSMURF2*
284   cells was significantly inhibited, and their transcripts were significantly inhibited in HUVEC-
285   K562 co-cultured cells. These may indicate that the down-regulate expression of these genes
286   would help inhibit the activity of ECs in the leukemia environment. To understand the role of
287   these genes *in vivo*, we analyzed the survival of leukemia patients when stratified by the
288   expression abundance. It was found that leukemia patients with lower expression of *DIDO*
289   showed better survival. Based on these findings, we focused this study on the *DIDO* gene.
290   DIDO plays an important role in mitotic progression and chromosome instability as it is a
291   component of the centrosome proteins and plays an essential role in spindle assembly [16]. It has
292   been reported that *DIDO* is related to chromosomal instability. *DIDO* gene may be a novel MSI
293   biomarker, as its mutation has a high concordance level with MSI-H status (microsatellite
294   instability high), based on research enrolled 1301 colorectal cancer FFPE (formalin-fixed,
295   paraffin-embedded) tissue sections [27]. There are increasing evidence showing that the *DIDO*
296   plays an important role in tumor onset and progression. In bladder cancer, the reduction of *DIDO*
297   mRNA resulted in increased apoptosis, reduced proliferation *in vitro*, and inhibited
298   tumorigenesis *in vivo*. The authors pointed that the potential mechanism of *DIDO* action might
299   involve SAPK/JNK signaling cascades [15]. In addition, in melanoma cells, *DIDO* was found to
300   induce the expression of integrin α, and promoting the attachment, migration, invasion and
301   apoptosis resistance of melanoma cells [17]. In this study, the transcript and protein abundance of
302   *DIDO* gene was inhibited in siRNA cell lines, which resulted in an inhibition of proliferation,
303   and an up regulation of apoptosis. This is consistent with the bladder cancer and melanoma.
304   To investigate the genes that are affected by *DIDO*, the DEGs between *shDIDO* and *shCtrl* cell
305   lines were analyzed. The ERK/MAPK signaling was significantly inhibited in both the HUVEC-
306   K562 co-cultured cells and *shDIDO* cells, the ERK5 pathway mediates apoptosis and
307   proliferation signaling in several kinds of tumor cells [25]. It was reported that the ERK5 was
308   regulated by phosphorylation and established a link between the CDK pathway during mitosis
309   [28]. On the other hand, the *cyclin D1* gene is a key step in cell proliferation, and it may be a novel
310   target of the ERK5 cascade [29]. In this study, we also found that the expression of cell cycle
311   genes, *CDK6* and *CCND1*, were down-regulated in *shDIDO*, which was verified by qRT-PCR
312   and western-blot. According to these results, there may be crosstalk between *DIDO*, *ERK5*,

313 *CDK6*, and *CCND1*, and these genes may work together to inhibit cell proliferation. However,
314 how these genes interact with each other still need further study.
315 As *DIDO* may act as a transcription factor [30], we screened for potential *DIDO* target genes that
316 down regulated in *shDIDO* cells. The IPA interaction network indicated that *DIDO* directly
317 interact with *WWP2*, *SRPK2* and *SRSF1*. *WWP2* (WW domain containing E3 ubiquitin protein
318 ligase 2) gene encodes a protein that play a role in the regulation of oncogene signaling pathways
319 via interactions with SMAD proteins and the tumor suppressor PTEN. *WWP2* could promote the
320 proliferation of gastric cancer cells in a PTEN-dependent manner, and its silencing will inhibit
321 proliferation and growth of gastric cancer cells [31], suggesting a vital role of *WWP2* in cancer
322 progression. *SRPK2* (Serine/Arginine-Rich Protein-Specific Kinase-2, SRSF protein kinase-2) is
323 up-regulated in multiple human tumors, and plays an important role in the progression and
324 metastasis of prostate cancer [32]. *SRSF1* (serine/arginine-rich splicing factor 1) promotes
325 proliferation and injury-induced neointima formation in vascular smooth muscle cells [33], and it
326 could promote tumorigenesis through regulation of alternative splicing in colon cancer [34],
327 glioblastoma [35], and other cancers. The overexpression of *SRSF1* could promote cell
328 proliferation and delay cell apoptosis during acinar morphogenesis in breast cancer [36].
329 According to these previously researches, *DIDO* may play roles by interaction with *WWP2*,
330 *SRPK2* and *SRSF1*.
331 In order to analyze the genes or epigenetic modification that may regulate the expression of
332 *DIDO*, the published ChIP-seq data in different leukemia cell lines (including K562) was
333 analyzed. There are multiple DNase-seq peaks near *DIDO*, presuming that there are regulatory
334 factors binding at the corresponding position. As shown in Figure 4 and Figure sS4, the *CDK7*,
335 *CDK8*, *ATF1*, *BCLAF1*, and *CBX3* had binding peaks at the transcription start site (TSS) near
336 exon 1 and exon 2. H3K4me3 and Pol II (POLR2A) bind to the site near *exon1* and *exon2* of
337 *DIDO* gene. The *EGR1*, *FOS*, *MAX*, *NCOR1*, H3K4me1, H3K27ac, *MED1*, and *EP300* had
338 binding peaks specifically at the TSS on exon1, and *CHD7*, *SIRT6*, *c-MYC* indicate binding
339 signals near the exon 2 TSS. This indicate that the transcript of *DIDO* gene was regulated by the
340 transcript factors and epigenetic factors. However, the correlation between these factors and the
341 *DIDO* gene needs further experimental verification.
342
Conclusions
344 In conclusion, the apoptosis and proliferation mechanism in leukemia endothelial cells that may
345 regulate by *DIDO* was summarized in figure 5. The ERK5 signal will be inhibited by the down-
346 regulation of *DIDO* in *shDIDO* cell lines, and the genes in ERK5 signaling may play roles in cell
347 apoptosis and proliferation, and regulate the gene transcription and translation. In addition, the
348 inhibited *DIDO* in HUVEC will indirectly inhibit the expression of *CDK6* and *CCND1*, which
349 will inhibit the proliferation of cells. The proliferation inhibition of endothelial cells may inhibit
350 the development of leukemia, and inducing cell apoptosis may become a therapy to treat
351 leukemia. The *DIDO* gene discovered in this study provides a theoretical basis for the

352 development of drug targets for leukemia. But it is also necessary to study the gene expression of
353 *DIDO* in leukemia patients.
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477

Figure 1

Figure 1 The KM-plot in Acute Myeloid Leukemia stratified by the expression level of DIDO (A), ZC3H18 (B), and SMURF2 (C).
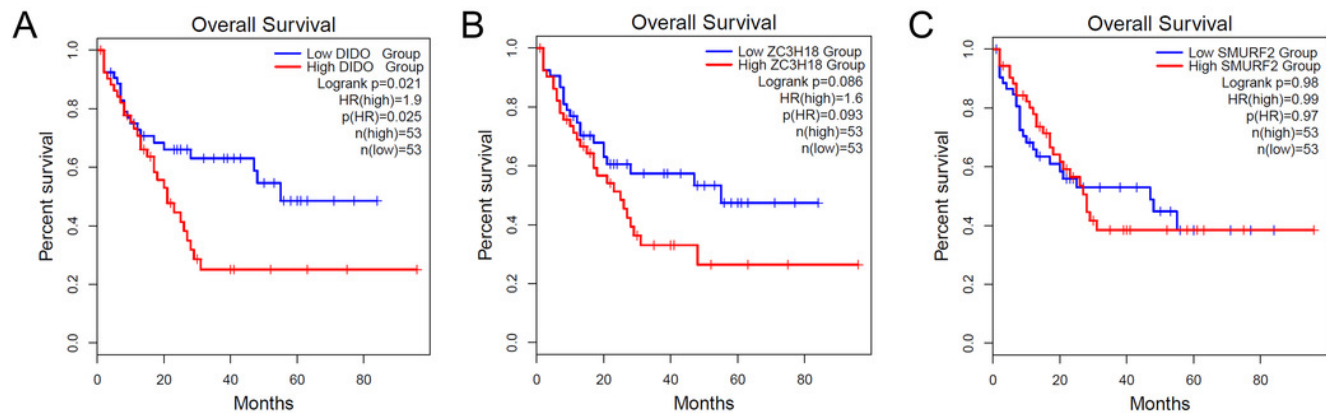
Figure 2

Figure 2. The function assays of *DIDO* gene.

A) The RNA transcript abundance of *DIDO* gene in *shDIDO* and *shCtrl* cells detected by qRT-PCR; B) Western-blot assay for the expression of DIDO protein; C) Cell proliferation pictures of *shDIDO* and *shCtrl* cell lines by Celigo; D and E) Cell count of *shDIDO* and *shCtrl* cell lines; F) Analysis of the area of blood vessel formation; G) Cell apoptosis ratio analyzed by FACS; H) Caspase3/7 activity assays.

A

B

C

D

E

F

G

H
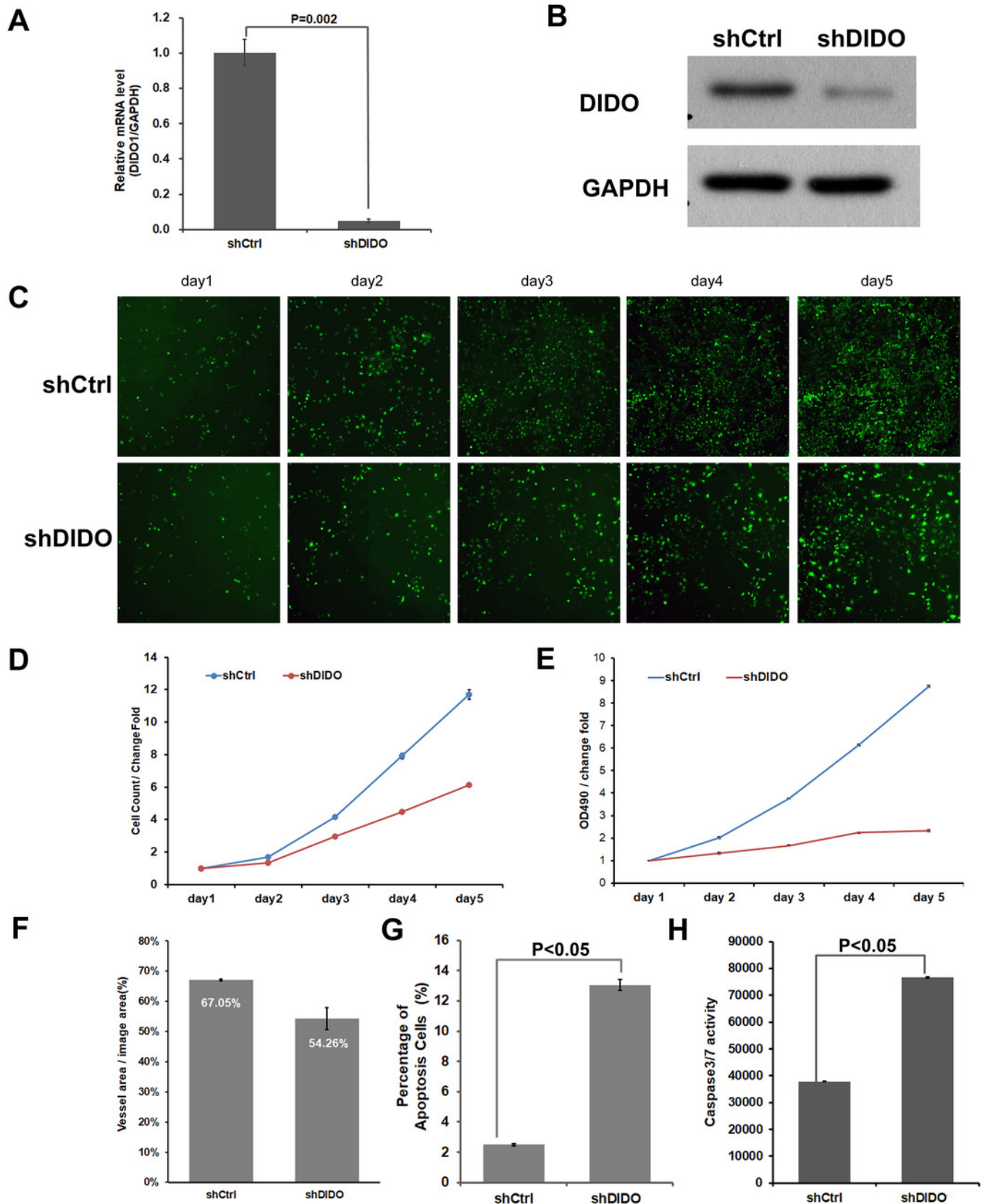
Figure 3

Figure 3 Analysis of downstream genes regulated by *DIDO*

A) The volcano map of the differently expressed probes in *shCtrl* and *shDIDO* cell lines; B) The enrichment of DEGs in the classical signal pathway; C) Disease and function heat maps show the expression changes of DEGs indifferent diseases and functions. Orange means the disease or functional state is activated (Z-score>0), blue means the disease or functional state is inhibited (Z-score<0), and gray means the disease or functional state is not determined (Z-score cannot be calculated); The disease or function is significantly activated if Z-score>2), and significantly inhibited if Z-score<-2. Significantly activated diseases or functions include: morbidity or mortality (Z-score= 6.734), organic death (6.709), etc.; Significantly inhibited diseases or functions include: cell viability (-5.369), cell survival (-5.349); D) Gene interaction network diagram shows the interaction network between molecules; E) The expression of *CCND1* and *CDK6* analyzed by qRT-PCR and Western-blot.
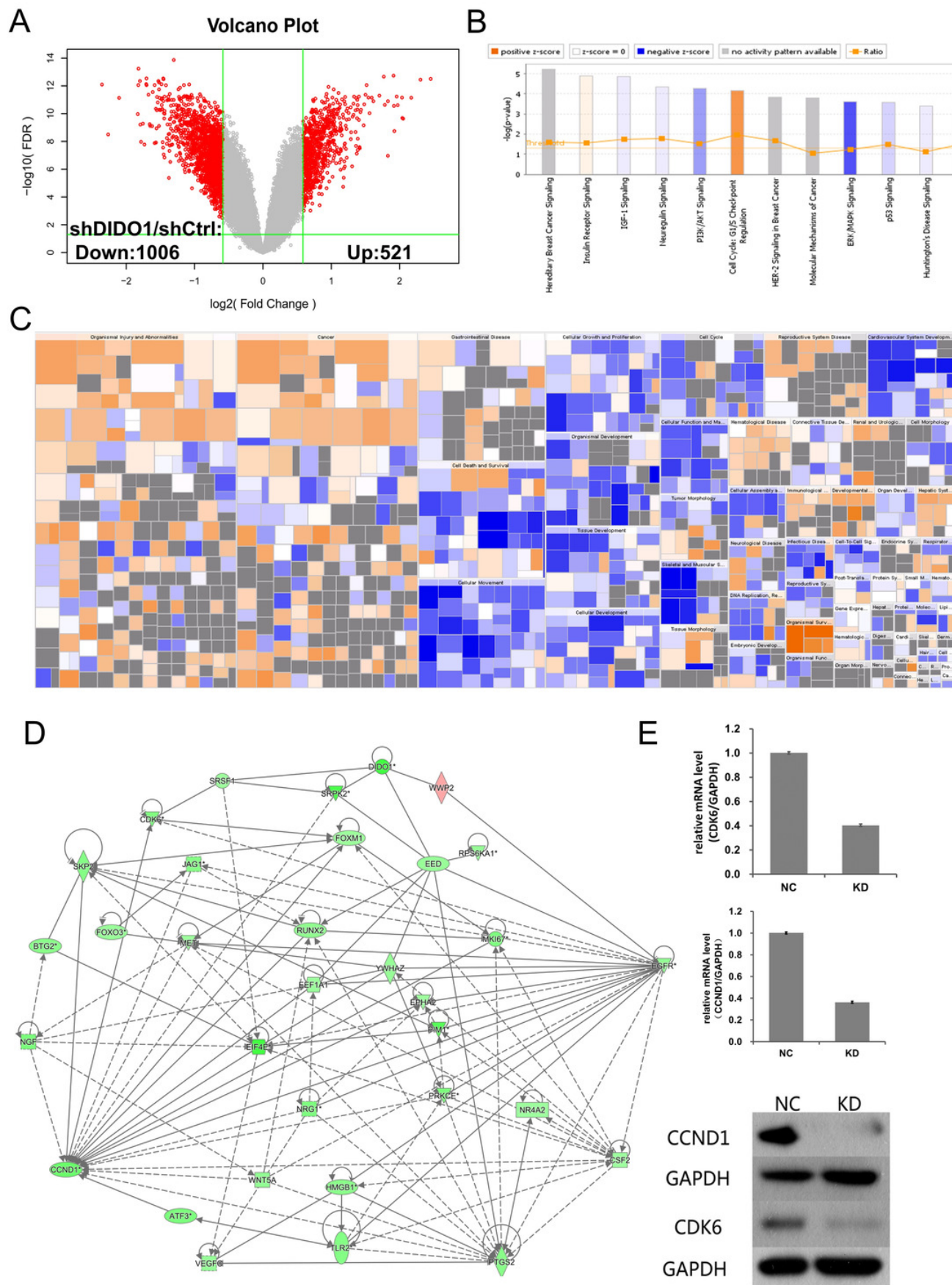
Figure 4

Figure 4 The transcript factors and epigenetic factors bind with *DIDO* gene analyzed by public data.
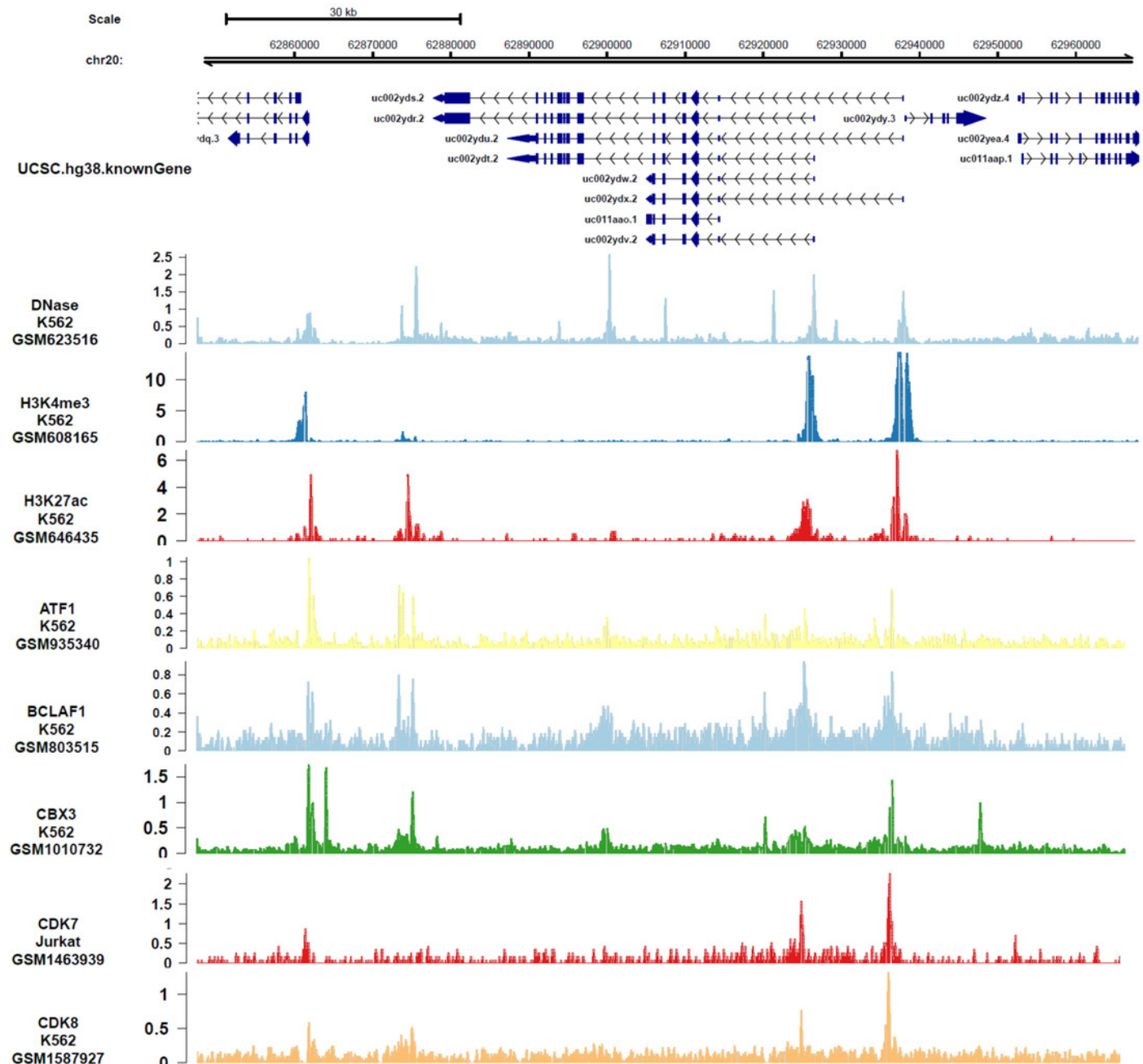
Figure 5

Figure 5 The apoptosis and proliferation mechanism in leukemia endothelial cells that may regulated by *DIDO.*

Green represents down-regulated expression of genes in *shDIDO* cells, and pink represents up-regulated expression of genes in *shDIDO* cells. The solid line indicates clarified relationship between genes; The dotted line indicates the relationship between genes needs further study.